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OBSERVATIONS ON REPRODUCTION IN CERTAIN PARTHENOGENETIC AND BISEXUAL NEMATODES REARED IN ARTIFICIAL MEDIA*

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INTRODUCTION

Nematodes present material for a wide variety of investigations. Not only is the group very incompletely known, especially the free-living species, but much also remains to be learned about their life processes. One of the fertile, outstanding possibilities for investigation relates to the remarkable features of reproduction presented by these animals. Maupas ('00) and others have pointed out the striking diversity in the methods of reproduction and the possibilities of this group in extending the knowledge of sex determination, origin of parthenogenesis, origin of bisexuality, and the nature of hermaphroditism. The development of methods whereby these animals can be maintained in pure cultures for indefinite periods adds to their value as material for critical study. In this paper, the writers present results of observations on cultures maintained continuously for more than three years. The data herein presented are general in character and the results of study of certain special problems will appear at a later date.

The writers wish to express their indebtedness to Dr. N. A. Cobb for the identification of the specimens used in this work and to Mr. A. L. Ford who, during the absence of the writers, maintained the cultures through the summer months of 1917.

METHODS OF CULTURE

In connection with the work on which this paper is based, the writers had occasion to use a number of methods for rearing nematodes in artificial media. Previous investigators devised means whereby certain free-living and parasitic nematodes could be maintained for a number of generations under laboratory conditions and some of their results were used to advantage in the present work. In some cases, previously known methods have been modified, while in others, new procedures were developed. It is obvious that, in order to study these animals

successfully, they must be reared under conditions other than those of the natural environment and the fact that they can be cultured in artificial media for indefinite periods of time makes it possible not only to observe continuously the details of activities and of life history but also to carry on a large variety of experiments. The writers maintained continuous cultures of *Diplogaster ærivora* and *Cephalobus dubius* for over three years. Cultures of the latter are still under observation and are all descendants of one original stock. Since the published methods of rearing the smaller nematodes appear in scattered sources, the more important ones will be given brief notice here. In addition, the methods employed by the writers will be discussed in detail.

Methods of Other Investigators

Conte ('00a, p. 374) cultured *Rhabditis monohystera* by employing as nutritive media "la colle de pâte trés epaisse les solutions de peptone et les tranches de pomme de terre. Ces cultures etaient faites dans des assiettes couvertes et, d'autre part sur porte-objets en isolant un femelle fécondée dont j'etudiais ensuite la descendance." He was also able ('00b, p. 376) to rear *Diplogaster longicauda* "dans la colle de pâte."

Metcalf ('03, pp. 93-98) reared a nematode of uncertain identity (*Rhabditis brevispina ?*), found in diseased corms, young stalks of *Crocus* and cuttings of *Petunia*, *Coleus*, and *Geranium* on plates of asparagus agar. Sterile agar was unsuitable, but a one per cent asparagus juice agar, inoculated with a *Fusarium* and the bacteria which occurred with the original stock of the nematodes and allowed to decay for about two weeks, was satisfactory after it had been heated, filtered, and sterilized. In this medium, sterilized eggs developed rapidly and normally. A certain fluidity of the medium was found to be desirable.

Potts ('10, pp. 443-446) readily cultured certain free-living nematodes (*Diplogaster maupasi*, *Rhabditis gurneyi*, *Rhabditis elegans*, *Rhabditis duthiersi*, *Rhabditis sechellensis*) in drops of nutrient media in watch-glasses closed with a vaselined glass cover. Solutions of "brown" and "white" peptone, used almost exclusively as the media, were allowed to putrify until a cloudy growth of bacteria had formed throughout them. In the presence of large numbers of bacteria, the nematodes thrived but, in sterile solutions, growth was suspended and eggs were deposited

only at long intervals. Potts did not record the strength of the peptone solutions—a matter of importance since, as will be shown later, an excess of peptone has a deleterious effect on the worms. Two or three successive generations were reared in a saturated solution of gelatin in water, and mature individuals were secured from eggs in "solutions of amides like tyrosin and leucin" but restriction of both growth and egg production indicated that these media were inferior to peptone. Beef infusion was also used to a very limited extent.

Oliver ('12) cultured an unknown nematode, found in the exudate about the genitalia of dead guinea pigs, in a medium made by inoculating a little of this exudate onto moist earth and slants of Musgrave's amœba agar, kept at a temperature of about 75° F. Successful subcultures were also made by using plain agar and ascites agar, plain agar and amœba agar giving the best results.

Johnson ('13, pp. 612-618) was able to culture *Rhabditis pellio*, a parasite of the nephridia of the common earthworm (*Lumbricus terrestris*), by methods somewhat similar to those used by Potts, Conte, et al. Filtered tap water or salt solution in watch-glasses to which were added certain food materials constituted the basis of the cultures. The watch-glasses were either covered individually by means of a vaselined glass cover, or else several, without covers, were placed in a humid chamber which gave a larger air space but minimized evaporation. The culture medium was replenished from time to time. Experiments with Witte's peptone showed that a nearly saturated solution quickly proved fatal to the nematodes and the same result accompanied weaker solutions until a 0.15 per cent strength was reached. Although very dilute, this solution proved satisfactory for one series of cultures, but afterwards it was almost uniformly unsuccessful. A one per cent. hay infusion was successful for only one series. Meat extract, decaying meat, a solution of urea, and a hay infusion sterilized and inoculated with soil bacteria were all virtually useless. Decaying earthworm had an advantage over peptone in nutritive value, but presented the disadvantages of requiring special treatment to prevent introduction of nematodes already present in the worm; of becoming increasingly opaque as decay advanced, thus rendering examination difficult; and of occasionally becoming foul smelling and densely clouded, causing the death of all of the nematodes. The best medium was prepared by removing the alimentary tract from freshly killed *Lumbricus terrestris* and placing the body in a test tube

which was then plugged with cotton-wool, heated in a steamer and kept at a boiling-point for about two hours, decanted, filtered, and inoculated with the bacteria which were associated with the worm in the soil. These bacteria were secured by allowing a fresh *Lumbricus terrestris* to decay in a small amount of water to which a little earth had been added. This medium also possessed the disadvantage of occasional development of bad odor and opaque scum, leading to the death of the entire culture.

Byars ('14) made use of a synthetic medium, Pfeffer's nutrient agar, in connection with some studies of the plant nematode *Heterodera radicicola* and found it suitable for the preliminary development of the worms up to their penetration into the tissues of experimental seedlings grown in the same medium. Most of the individuals which remained outside of the root of the seedling died within a few days, although a few remained active for more than a month. The latter, however, did not undergo their normal development into adults. It will be shown later that this medium is much more successful for certain other species.

Merrill and Ford ('16, pp. 121, 126) found that *Diplogaster labiata* flourished in water cultures to which had been added portions of the macerated bodies of the beetle *Saperda tridentata*—the host of this nematode. They state that "Different substances were tried with varying success, but macerated beetles placed in water seemed to be the most satisfactory" but these "different substances" were not specified. They also reared *Diplogaster ærivora* in similar preparations.

Methods of the Writers

Equipment

The writers had occasion to employ a variety of culture methods demanding a corresponding variety of equipment. Most of the cultures were maintained in cells constructed as follows: Cylindrical glass mounting-cells with ground edges were sealed to ordinary glass microscope slides by means of pure vaseline and covered with a circular cover-glass, the diameter of which was slightly greater than that of the glass mounting-cell. The cover-glass was also sealed on with vaseline. For stock cultures, the most suitable glass mounting-cell was found to be one having a diameter of 20 mm. and a height of 10-15 mm., preferably 15 mm. These tall cells made it possible to maintain a greater volume of the liquid culture medium, thus providing for a larger stock and, more im-

portant still, minimizing the danger of cultures drying out since it was discovered that they could not be sealed tightly but demanded at least a small opening at the top. They offered the disadvantage of being too high for examination with anything but very low powers of the compound microscope. It was possible, however, to use the binocular microscope, and since there was little occasion to use the stock cultures for purposes other than maintenance, this disadvantage was of little consequence.

Cultures maintained for experimental or detailed observational work were kept in glass mounting-cells of another size, viz., diameter, 20 mm., height, 6 mm. Such a cell surrounded a space large enough to enclose a drop of the culture medium without involving the danger of the latter coming into contact with the vaselined edges of the cell, a precaution which had to be observed rigidly. That height also permitted examination with the ordinary low powers of the compound microscope without removing the cell from the supporting slide.

Many of the glass mounting-cells used in this work have walls 1 mm. thick. Cells having walls of 2 mm. thickness were found more advantageous, especially for stock cultures, owing to the greater vaselined area in contact with the slide, thus minimizing the danger of the watery medium leaking out and permitting the culture to dry up. The thinner cells were in more constant use owing to the difficulty of obtaining the thicker ones, and, if care was taken to thoroughly vaseline the ground edges and press them down onto the slide, they were quite satisfactory.

In special cases when greater space or capacity was desired, small stender dishes were used. A limited use was made of a group of glass mounting-cells sealed to the bottom of a Petri dish. For rapid observation of isolated individuals, Petri dishes alone were used for one series, large drops of the nutrient medium retaining their identity when placed on the dry, clean glass but not in contact with each other.

At times, culture slides of two kinds were used in place of the glass mounting-cells mentioned above. One form is the heavy plate glass, 76 x 26 mm. slide, 5 mm. thick and with a central, circular depression 4 mm. deep, and covered by sealing on a circular cover-glass with vaseline. This form of receptacle was satisfactory in many respects but was not usable for individual cultures because of its depth. Furthermore, it is more expensive than the glass mounting-cell. The other culture slide is the ordinary 76 x 26 mm. form, having a central, shallow

concavity with maximum depth of about 1 mm., closed by a vaselined circular cover-glass. This type is useful for individual cultures.

Needles for transferring eggs, larvæ, and adults from one culture to another were in constant use. These instruments were made by setting into a small handle a fine needle, the point of which had been bent into a small recurved hook. It was found convenient to have several of these needles whose points were bent at different degrees. Ordinary insect pins of sizes 0 and 00 were used to some extent but the most useful form was the Japanned steel "Minuten Nadeln" commonly used for pinning minute insects. These pins made especially useful needles because of their very small diameter and extremely fine points, thus facilitating their manipulation under the high power of the binocular microscope and their use in transferring the minute nematodes.

Transferring brushes, which for part of the work were used instead of needles, were made from small camel's hair brushes by carefully cutting out the brush until a very small central tuft of 3-5 hairs remained. Such a brush was often used for transferring eggs.

Pipettes of the ordinary form were in common use for handling the various fluids employed in the work. In order to avoid contamination of the cultures, it was necessary to keep these pipettes properly labeled and thoroughly clean. A special form of pipette for removing specimens or for removing deteriorating culture fluids was made by drawing out in a flame the open end of an ordinary straight pipette until the opening was only about 0.3 mm. in diameter.

Media

In culturing nematodes, it is necessary to use some substance which will serve directly or indirectly as food for the animals. The writers have tried out a number of substances some of which have been distinctly successful. Since the same substances were not in every case employed for both of the species reared, the culture methods of each will be discussed separately.

Practically all of the culture media were more favorable under conditions of dilution, in fact, some of the most successful ones demanded extensive dilution, otherwise they were inimical to the worms. Distilled water was uniformly used, thus eliminating the danger of contaminating the cultures from that source, as would be the case with tap-water. Preliminary experiments with both *Cephalobus dubius* and *Diplogaster*

ærivora showed that all stages of the life history lived for some time, several days in many cases, in distilled water alone, and when a small amount of nutritive substance was added, the medium became suitable for continuous culture. Apparently, the usual toxicity of ordinary distilled water had no deleterious effect on the worms.

Diplogaster ærivora.—Since the original stock of this species was found in the eggs of grasshoppers, the yolk of these eggs was at first used as a medium and, as might be expected, gave good results. Eggs from a number of species of grasshoppers appeared to be of equal value. Owing to the occasional difficulty or inconvenience in getting grasshopper eggs, the eggs of certain Coccinelidæ, of the Colorado potato beetle, of certain unidentified insects, and the ovaries of grasshoppers were successfully substituted. Furthermore, the softer tissues of young grasshopper nymphs, army worm pupæ, variegated cutworm pupæ, Hessian-fly pupæ, and pupæ of a number of other insects were found to constitute good media when prepared in the proper way. It is very probable that a wide variety of insect eggs and tissues would serve this purpose equally well.

The procedure in the use of the above mentioned substances depended upon the kind of culture desired. Most of the stock cultures, as well as the majority of the life history cultures, were maintained in the glass mounting-cells already described. When grasshopper eggs were used, they were first carefully cleaned externally and stripped of their shells, since fragments of the latter when present tend to render the examination of the culture more difficult. The contents were placed in a small amount of distilled water and thoroughly triturated in order (1) to distribute more uniformly the yolk throughout the culture and render it more readily available to the nematode, and (2) to facilitate the examination of the material for evidence of previous nematode infestation, thus aiding in the maintenance of pure cultures. This precaution is necessary since small nematodes often occur on the surface and apparently within some of these insect eggs and pupæ. The writers found them in connection with Hessian-fly puparia, both on the surface and apparently in the pupæ, although in the latter case there is the possibility that they might have been included by manipulation. However, Marchal has reported Osborn, '98, p. 41) nematodes in the puparia of this insect. Insect tissues mentioned in a foregoing paragraph were sometimes used in a

similar way, removing, of course, all fragments of the integument. The proper amount of the diluted nutriment was placed in the glass mounting-cell which already contained the appropriate amount of distilled water.

The method of adding the food materials thus prepared was determined by the purpose of the culture. For stock cultures, two or three drops from a pipette were added every 3–5 days to the 1–1.2 c.c. of distilled water in the mounting-cell. A larger amount of food could be added without serious results to the organisms but it was not needed for nutritional purposes, and it had the disadvantage, at least in certain cases, of tending to increase the opacity of the culture.

When there was any occasion for using large quantities of the material for a medium, good results were secured by putting sterilized soil in a covered stender dish, moistening it liberally with distilled water, and transferring to it a pupa or other food object which was then perforated and inoculated with the nematodes from a stock culture. These cultures lasted for considerable periods of time, developing nematodes in quantities.

While the above-described methods were satisfactory, it was inconvenient in the maintenance of a long series covering different seasons of the year to keep a constant supply of the food materials at hand and to have a continuous stock of any one kind for experimental purposes. For that reason, a search was made for some food which is approximately constant in composition and easily available at any time. Among other things, the yolk of hen's egg was tried and found successful. Experiments with different amounts of this substance showed that, in a culture containing 1–1.3 c.c. of distilled water, the nematodes thrived when the quantity represented by three or four dippings of the points of ordinary forceps into the yolk was added. Almost any larger amount may be used but increased opacity and the occasional undue putrefaction make it undesirable. Amounts of food represented by one or two dippings of the forceps were, in general, found to be distinctly inferior in results and evidently represent too poor a culture. A single hen's egg supplied food material for a number of days. A small opening, approximately 1 cm. in diameter, was made through the shell and the adjacent albumen allowed to escape. Sterile forceps were used to lift out the desired quantities of the yolk and then the opening in the shell was sealed over with a gummed label or piece of gummed cloth and the egg kept in a cool

place. When needed again, the gummed covering was removed, the desired quantity of yolk secured, and the opening again sealed. The amount of yolk adhering to the points of forceps is variable but the method was found to be both rapid and practical in the operation of ordinary cultures.

Liebig's extract of beef, diluted to different degrees with distilled water, was tried as a nutrient medium and found to be usable so far as maintaining the animals was concerned but its partial opacity impaired its usefulness.

Since Byars ('14, p. 323) found Pfeffer's synthetic agar useful in culturing certain nematodes parasitic on plants, it occurred to the writers to experiment with it as a possible medium for rearing *Diplogaster ærivora*. The medium used was as follows: calcium nitrate, 4 grams; potassium nitrate, 1 gram; magnesium sulphate, 1 gram; potassium dihydrogen phosphate, 1 gram; potassium chloride, 0.5 gram; ferric chloride, trace; distilled water, 6 liters; and agar, 12 grams. The formula used by Byars calls for powdered agar but the writers used the shredded agar cut up into small bits. This fluid was used directly from the stock solution without change and, while it appears from all the trials that it is somewhat inferior to certain other media used, nematodes were maintained in this solution for long periods of time. According to the experience of the writers, cultures in this medium, when properly cared for, are fairly satisfactory, but since there was some evidence of inferiority, parallel series of tests were carried on for 20 days, comparing the relative merits of Pfeffer's solution and hen's egg as media. Using the rate of development and reproduction as indices of the value of the media, four of the five series showed evidence of the superiority of the hen's egg, one of the five showing a slight advantage for the Pfeffer's solution.

Maceration cultures used by the writers have already been described and it seems very probable that, barring the danger of contamination, they might be effective and that a number of different animals could be used as sources of food supply. Mr. A. L. Ford reared *Diplogaster ærivora* for one year on the macerating bodies of adult *Saperda tridentata* and *Hydrophilus triangularis*. He also found it possible to use macerated beef but the cultures proved somewhat unsatisfactory because of the offensive odor. This nematode was found by Merrill and Ford ('16) parasitizing termites and Mr. Ford has informed the senior writer that he tried the macerating bodies of these insects as a medium but found it

unusable since a mould almost invariably appeared in the cultures, filling them with threads and obscuring the entire preparation. The writers also tried macerated termites with similar lack of success from the same cause.

Cephalobus dubius.—Maupas ('00, p. 611, 613) found some difficulty in culturing the parthenogenetic nematodes, including this species, stating that "elles se prêtent mal à des cultures en grand." However, the writers have been able to culture *Cephalobus dubius* in countless numbers at any time. Since studies of this species were not begun until cultures of *Diplogaster ærivora* had been in progress for about two years, the methods which had proved most satisfactory were tried for *Cephalobus dubius* and were likewise found to be successful. Several series of cultures were maintained in Pfeffer's solution but many of the life history studies were made using the hen's egg preparation. Both media were used as described in the preceding section, and both proved suitable for prolonged cultures, indications pointing to the hen's egg being slightly preferable. Grasshopper eggs, Colorado potato beetle eggs, and pupæ of Hessian-fly also proved suitable but were not always readily available.

As has been mentioned, other workers made some use of peptone as a nutrient medium for the culture of nematodes. After a number of trials with different strengths of peptone, a solution was found which has proved thoroughly satisfactory and, at the present writing, cultures of *Cephalobus dubius* have been running in this medium for months, without any indication of weakness. The strength of solution used by Potts is not given in his paper. Johnson discovered that strong solutions are inimical to the nematodes and that dilution to as low as 0.15 per cent. was necessary, this strength proving satisfactory for one series of *Rhabditis pellio*. The writers have had a similar experience, finding that strong solutions kill *Cephalobus dubius* in a short time but that very weak ones are quite efficient. In the work on which this paper is based, 0.8–4.6 per cent. solutions were used. In fact, it was found unnecessary to make up solutions of definite strength, except for special studies, since the stock cultures could be kept in flourishing condition by occasionally (every four to six days) adding to the water in the mounting-cell containing the nematodes the small amount of peptone which would adhere to the point of a common dissecting needle. This method of measure was, of course, subject to variation but within its limits the

varying quantity seemed to produce no appreciable effect on the cultures and indicated a range of over five per cent. within which the solution is suitable. The exact maximum and minimum dilutions for this species were not determined. It should be mentioned in this connection that even longer intervals between "feeding" were possible if necessity demanded. The writers had one peptone culture made up in the above-described way which existed for over four weeks without renewal of the food, the nematodes thriving and reproducing during the whole period.

As stated on a preceding page, Potts found that it was necessary for peptone solutions to putrefy until a cloudy growth of bacteria had developed and that it was "only in the presence of great numbers of bacteria, or the substances formed by them, that the nematodes thrive so well." In cultures of *Cephalobus dubius* maintained by the writers, it was not necessary to develop any putrefaction of the peptone solution but the dry peptone was added directly to the water of the rearing cell. When new cultures were necessary, distilled water was placed in the cell, a small amount of peptone on the end of a dissecting needle was transferred to it, and the nematodes then introduced. This procedure was followed a great many times and without any appreciable diminution in the activities of the animals. Furthermore, cultures made up in this way have been kept for a month without developing cloudiness and the nematodes grew and reproduced rapidly. Since no attempt was made to keep these cultures sterile, bacteria did develop in them to some extent and it may be that in those cultures which remained perfectly clear a limited amount of bacteria was present. It should be mentioned in this connection, however, that a distinct cloudiness did appear in some of the cultures after standing several days, developing gradually into a distinct brown color. This occurred rather commonly in summer and was possibly due, in part at least, to the accumulation of bacteria. But, with this particular nematode, the development of the brown color was accompanied by a general deterioration of the culture and necessitated either a removal and renewal of the greater part of the culture medium or a transferrence of the nematodes to a new solution. It would then seem that *Cephalobus dubius* requires only a very low development of bacteria in the culture medium, if it is required at all, and that an undue development of the cloudiness is usually detrimental.

General Procedure

Certain general methods of procedure were found to be preferable, and, in some cases, necessary to the maintenance of the cultures. In stock cultures reared in glass mounting-cells, the optimum amount of medium was found to be about two-thirds to three-fourths the capacity of the cell. Most of the cells were kept approximately half filled. Smaller amounts could be used but involved the danger of the cell drying up between observations.

Early in the work, it was noticed that when a stock culture was sealed over completely with a vaselined cover-glass unfavorable conditions became established and the culture would often die out. Experiments were performed to determine the effect of the presence or absence of an air-space above the medium and it was definitely demonstrated that such a space must be provided, hence the above-mentioned procedure of never filling the cells more than three-fourths full. Furthermore, it was found that even with an air-space provided, unfavorable conditions would develop in a few days if the cell was sealed air tight. This situation was avoided by slipping the cover-glass back so that a small crescent-shaped opening between the edge of the cover-glass and the walls of the mounting-cell was produced. This method was entirely satisfactory although it involved a certain loss of the water by evaporation, thus necessitating an occasional restoration to the original level in the culture-cell. The exact reason for this demand for ventilation is not known. Johnson ('13, p. 612), who also discovered the necessity for providing against completely closed cultures, thought that it was necessary to permit the escape of gaseous decomposition products originating in the culture medium. It is true that in cultures made from such materials as yolk of hen's egg, insect eggs, and ovaries of grasshoppers, decomposition products were formed since they tend to quickly develop offensive odors. On the other hand, Martin ('13, pp. 94-97; 143) has pointed out the indispensability of free oxygen to the development of the embryos of nematodes and it may be that the demand for oxygen is the explanation. Since many of the culture media used were undergoing decomposition and since this process draws upon the available oxygen, it is possible that the oxygen supply in one of the sealed culture-cells, having either a small air-space or no air-space at all, would soon be reduced to an unfavorable extent.

Occasionally, even the partially closed stock cells, especially those containing peptone, developed not only a cloudy appearance but also a brown color which gradually increased in intensity. The specific cause of this color is not known but it was evidently a result or an accompaniment of the general activity of organisms in the cell and almost invariably led to the death of the nematodes if the conditions were allowed to continue. Thus it was necessary either to transfer nematodes to an entirely new culture cell or else remove the old medium and replace it with new. To accomplish the latter, the point of a finely drawn pipette was thrust just below the surface of the medium in the stagnant cell and the liquid very slowly removed. In this way, the eggs, young, and adult nematodes which are always at the bottom were but little disturbed and few of them lost in the process. Then the old medium was replaced with distilled water and a small amount of peptone added. If necessary, a number of the nematodes can be transferred to new culture-cells by means of the transferring needles already described. In stocking a new culture, only a few nematodes need be transferred.

In studies of the reproduction, development, growth, and activities of these nematodes, it was necessary to isolate individuals or eggs in order to follow definitely and accurately the sequence of events. To accomplish this end, cultures of a different type were used. The culture-cell was made up in the same way as those for the stock cultures except that it was necessary to use a cell not over 5 mm. high. A single drop of distilled water from a pipette was centrally placed in the bottom of a clean, dry cell. This drop, if carefully placed, retained its integrity. If the drop spread and came into contact with the edges of the cell, it was discarded. To this drop of water was added a tiny bit of the food material (the quantity which will just cling to the extreme point of a dissecting needle) and the nematode or the egg transferred to it. Since these single-drop cultures have a large air-space compared with the bulk of the fluid and since they must be examined frequently in connection with the recording of data, these cells were kept tightly sealed and in that way the danger of drying up was avoided. During the microscopical examination, the whole cell was removed from the slide, thus leaving the culture drop intact. It was necessary to guard against undue evaporation during the exposure of the drop and all losses had to be replaced. Transference of the nematodes, young or adult, was easily accomplished by means of the needles already described. Eggs were

transferred by the same means and likewise by the drawn out pipette already described. The latter method was advantageous in transferring a number of eggs at a time since they could be massed together in the culture-cell and then drawn up in the pipette with a relatively small amount of liquid. Furthermore, eggs were transferred by means of the brush described in an earlier paragraph but this method was inferior to the ones discussed above.

Starting Cultures from New Stock

Since *Cephalobus dubius* has been found but once and the entire series of cultures is from the original stock, the writers have had little experience in transferring this species from the conditions of nature to those of the culture-cell. However, *Diplogaster ærivora* has been brought in from outside conditions and established in cultures a number of times. New stock used by the writers usually came from grasshopper eggs and was secured by breaking up the eggs in culture-cells, diluting the yolk with distilled water, covering the cell in such a way as to leave an air-space and a ventilating opening, and putting it aside under temperature conditions of about 68-77° F. Often, after a few days, this nematode would appear in the culture whence it could be transferred to a more dilute medium of the same kind and then later to a different medium, if desired. Mention has already been made of the fact that Merrill and Ford ('16) found this nematode parasitizing termites. Mr. Ford has informed the senior writer that he could almost invariably secure a culture by the following method: A number of termites are killed and placed on the surface of water saturated soil in a small container. After some hours, nematodes usually emerge from the heads of the termites and continue their activities on the moist surfaces of the dead insects. In a few days, the disintegrating bodies are often swarming with the worms. Then a number of them are removed to a culture-cell containing the desired medium. Of those first transferred, the majority may die but usually a few will survive the change and reproduce, starting a new stock. Evidently, in a parasitic form, the transition from the body of the termite to the conditions of a culture-cell is a severe one but the immediate progeny of those which persist flourish in the new medium and thereafter maintenance is simple. This mortality of the parasitic nematodes, when removed to cultures, confirms, in part, the experience of Johnson ('13, p. 613) who frequently had great difficulty in starting cul-

tures of *Rhabditis pellio*, although, when once started, the difficulties of maintenance were considerably reduced.

Temperature Conditions of Maintenance

While no definite experiments have as yet been made to determine the exact maximum and minimum temperatures for these nematodes, certain observations are worthy of mention here. Temperature records for rooms where some of the cultures were kept showed that both species withstand successfully a considerable variation of temperature. In one room, the daily maximum-minimum records sometimes showed a difference of as much as 33° F. Such variation usually occurred during the winter months when the room temperature occasionally dropped from 74° to 42° F. Under these conditions, daily variation of 15-25° F. were common and a minimum temperature of 40° F. for a limited time (4-6 hours) apparently had no serious effect on the cultures. In this same room during the summer months, the variation was considerably less but both maximum and minimum temperatures were much higher, e.g., 92° and 84° F. It was found that when the room temperature rose much above 80° F., cultures began to show signs of weakness.

In cultures of *Cephalobus dubius*, the nematodes began to die under conditions of 90° F. These cultures were removed to an underground concrete cave where an almost constant temperature of 80° F. was maintained and evidence of strengthening was apparent. During the following thirty days (July), when the temperature in the cave gradually approached 87° F., the cultures showed increasing signs of weakness and ultimately maintenance became difficult. In the following month, when the temperature began to fall, signs of strengthening were evident as soon as 80° F. was approached. When 78° F. was reached, the cultures were soon flourishing again. In other respects, the conditions of rearing were the same throughout this period. Evidently, the maximum temperature for this species is near 90° F. and the optimum below 80° F. At the present time, stock cultures are thriving under laboratory conditions of 65-75° F.

Preliminary studies with reference to the influence of temperature upon *Diplogaster œrivora* were conducted by means of an air conditioning machine having two large breeding chambers in which constant temperatures (within very narrow limits) of 80° and 90° F., respectively, were maintained. These nematodes could not be reared in the 90° F.

chamber. Cultures submitted to that temperature soon showed decrease of activity, suspended reproduction, and early death. Fresh cultures reared in the 80° F. chamber and transferred to the 90° F. chamber suffered the same fate. Nematodes reared in the 80° F. chamber also exhibited some decrease in activity but continued to live and multiply, producing four generations in forty-three days. Cultures, kept under greenhouse conditions at a mean temperature of about 75° F., increased more rapidly than did those within the 80° F. chamber and the rate of development was greater, five generations being produced in forty-three days. Still other cultures were maintained in the laboratory under a variety of temperature conditions, and, according to the experience of the writers, 65-75° F. is favorable for rearing *Diplogaster ærivora*.

PARTHENOGENESIS IN *CEPHALOBUS DUBIUS* MAUPAS

Distribution

Cephalobus dubius is evidently a cosmopolitan species. Maupas ('00, pp. 555-556) states that it is very common in Algeria and that he found it in a sample of red earth collected in the environs of Tananarivo, Madagascar. In these localities, the nematode lives in rather poor earth and is said to withstand long desiccation and to revive when the moist conditions are re-established. Dr. N. A. Cobb states in a letter that it occurs in various parts of the United States and is found in moist situations, feeding upon animal matter. He also states that on several occasions it has been found on the surface of various insect eggs, and that the young withstand desiccation.

Source of Material

The original stock for all of the writers' cultures was secured accidentally in connection with the study of another nematode. In a preliminary experiment to determine whether *Diplogaster ærivora*, a species found feeding on the contents of grasshopper eggs, has the ability to penetrate insect eggs or whether it depends upon some other agency to provide the means of entrance, ordinary loam, sterilized by steam, was placed in a jar. Grasshopper eggs, cleaned of all soil particles but not subjected to special treatment, were introduced into this soil and specimens of *Diplogaster ærivora* in water added to the adjacent soil. When examined later, the eggs were found broken and deteriorating.

Under magnification, nematodes were seen in the egg contents and were transferred to culture-cells. It soon became evident that they were unlike *Diplogaster ærivora*, and when submitted to Dr. N. A. Cobb for identification they proved to be *Cephalobus dubius* Maupas. The original source is thus in doubt, but since the soil in the jar had been sterilized, it seems very probable that this nematode was carried into the culture on the surface of the grasshopper eggs, either as an egg or in some subsequent stage of development. Since there is evidence that at least the immature individuals of this species can successfully withstand desiccation, it is possible that they might have occurred on the surface of the insect eggs in that state, resuming activity when introduced into the moist conditions of the experiment.

Parthenogenesis

Maupas ('00), in his extensive study of reproduction in nematodes, found seven species in cultures of which he saw no trace of males. These seven species, distributed among six different genera, included *Cephalobus dubius*. He was inclined to admit the possible occasional occurrence of males but states that if they do appear they must be very rare.

Since the beginning of this work, the writers have watched carefully in the large number of successive generations for any appearance of males but in vain. Mature individuals, isolated in culture-cells, always deposited eggs. Similarly, isolated eggs or young that attained maturity always produced individuals which deposited eggs. Maupas expressed uncertainty as to the constant absence of males because of the fact that, in his studies, the greater part of the apparently parthenogenetic nematodes "se prêtent mal à des cultures en grand" and that with one exception he was unable to examine very large numbers. That uncertainty does not seem so significant since the writers were able, with their methods of culture, to get this nematode in almost any quantity and a very large number was examined for the possible appearance of males. Since the nematodes were, in part, maintained under certain differences and variations of food and temperature, it would seem that if males ever do appear in this species they must be extremely rare and apparently must develop under conditions of culture different in some unknown respect from those of the writers. Cultures of this species are still under observation in order that this matter, among others, may be more thoroughly tested. If it is later discovered that males do occasionally occur,

it will be interesting to determine whether they have all of the activities and functions of the sex or whether they resemble the imperfect males which occasionally occur in several of the hermaphroditic nematodes.

As Lankester ('17, p. 504) points out, parthenogenesis signifies "an exceptional and historically super-induced modification of the normal process of sexual reproduction or gamogenesis in which the female gamete or egg-cell does not unite with a male gamete or sperm-cell to form a 'zygote,' but proceeds to develop independently." It is a well known fact that protandric hermaphroditism ("Syngonism") is common among the free-living nematodes and Cobb ('15, p. 95; '16, pp. 198–199) suggests the desirability of re-examining the supposedly parthenogenetic forms to determine whether some of them are not actually "syngonic." This suggestion was made on the basis of a study of a series of syngonic free-living nematodes in which the spermatozoa present were smaller and smaller until they reached the optical limits of present instruments and he finds himself unable to assert the non-existence of spermatozoa in certain nematodes merely because he has not succeeded in finding them. This suggests that since both the parthenogenetic and the hermaphroditic nematodes have the unmodified form of the female, the former may, at least in some cases, possibly be instances of masked hermaphroditism.

The writers have not thus far made any microscopical examination of the gonads to determine the above-mentioned point and no comment can be made upon it except that the complete absence of males in continuous cultures maintained for over three years seems to indicate the parthenogenetic method of reproduction. If the animal were hermaphroditic, it would necessitate that every egg produced be fertilized and that fertilized eggs invariably produced hermaphrodites in order for the results of the writers' cultures to have been possible. Furthermore, as Maupas and others have pointed out, the hermaphroditic forms show an unbalanced relation between the number of spermatozoa and ova produced and the unfertilized ova do not develop but disintegrate after they are laid. In the case of *Cephalobus dubius*, all of the eggs laid were capable of development. Also, in the majority, if not in all hermaphroditic nematodes, males appear even though they may in some species be rare and imperfect in their sexual functions. At present, there seem to be no grounds for considering *Cephalobus dubius* other than a parthenogenetic form.

Oviposition

In order to obtain data on the reproductive capacity of the females, oviposition records were made as follows: Eggs, isolated from vigorous stock cultures, were placed individually in a single drop of distilled water in a glass mounting-cell. Frequent observations were made and the time of hatching carefully noted. A trace of yolk of hen's egg was then added and daily observations continued until the death of each individual. When oviposition began, the number of eggs appearing in the cell each day was recorded and the female transferred to a new culture, thus avoiding the labor of removing the eggs from the cell.

Cultures of thirty-six different individuals, representing a number of successive generations, were carried through the life cycle to cessation of oviposition and subsequent death and showed considerable variation in the length of the egg-laying period. These cultures were all maintained under the same conditions with respect to food but the temperature was that of the laboratory and subject to an average daily variation of about 18° F., the extremes being approximately as follows: minimum, 40-58° F.; maximum, 66-76° F. Temperature was the only factor which varied to any extent and the precise influence of this factor was not determined. Under these conditions, the duration of the egg-laying period was found to have a variation of 6–44 days, the average being about 16 days. This variation seems surprisingly large and leads to the suspicion that those nematodes with a short egg-laying period are individuals which, for some reason, had their normal existence shortened. But, since no account was taken of those individual life histories which were ended at a time when the egg production rate was high and only those which showed an acceleration followed by a subsequent retardation were considered, it does not appear that all such instances can be so interpreted. It should be stated that the maximum oviposition period of 44 days given in this set of records is much higher than any of the others, the next lowest being 31 days.

Egg-laying, when once initiated, continues uninterruptedly until its decline at the end of the life of the individual. None of the records showed any evidence of definite periods of cessation of egg-laying followed by active resumption of oviposition. Only two instances of 24–48 hour periods with no oviposition appears in the records and these occur at the end of the life of the nematode. The eggs are laid singly and at a

varying rate depending upon the age of the parent. The most characteristic feature of the records on all individuals presenting any evidence at all of a normal life cycle is the rather gradual increase in the daily rate of egg-laying during the first part of the oviposition period and the more or less gradual diminution in the last part. In every case, the maximum daily number of eggs deposited appears well within the oviposition period, ordinarily near the middle. Usually the initial and concluding daily rates are very slow, the latter often ceasing completely before the death of the nematode. In a few individuals only was the initial daily record above 10. The increase and decrease of the daily rate were not regular for any of the individuals studied. The maximum production of 27 eggs in one day occurs in the record of nematode No. 36. A few other records are close rivals. According to Maupas ('00, p. 560), "Par une température de 20° c., le maximum d'œufs pondus, dans les vingt-quatre heures, est de 12 à 13." It is evident that the daily rate of egg production is, at least at times, much higher than Maupas' record would indicate.

The total number of eggs per individual varied from 33 to 285, the average being 139. In general, those nematodes which lived longest and thereby had the greatest oviposition period produced the largest number of eggs but, in the writers' records, no constant relation exists between the duration of the oviposition period and the total number of eggs. For instance, nematode No. 55 produced 101 eggs in 9 days but nematode No. 79 deposited only 77 eggs in 13 days; nematode No. 24 produced 270 eggs in 44 days but nematode No. 85 produced 285 eggs in 30 days. Maupas ('00, pp. 561-562) reported a maximum of 415 eggs deposited over an egg-laying period of about 4 months.

In addition to the above, another set of oviposition records were made from cultures kept in an underground, concrete cave, where, during the period involved, the daily temperature variation was within 5° F., usually within 4° F. The average maximum temperature was 77.8° F., the variation being 75-79° F., and the average minimum temperature was 73° F., the variation being 71-76° F. While the temperature was not completely controlled, the fluctuation was small and the results are of interest when compared with those already described. The remaining conditions of the cultures were like those of the other series with the exception that peptone was used instead of the yolk of hen's egg. Six individuals were

successfully carried through their complete life cycle and the egg-laying period varied from 15 to 39 days. The total number of eggs deposited by each individual varied from 78 to 234. This series of cultures also presented the characteristic increase and diminution of the daily rate of oviposition which has already been described. The lack of a definite relation between the length of the egg-laying period and the total number of eggs deposited was also apparent although the general tendency for the longer periods to be accompanied by the larger number of eggs was evident. Owing to the difference in the numbers of individuals studied in the two series, a more detailed comparison is not possible.

After oviposition, the development of the embryo goes on rapidly and within a few hours the egg, under magnification, shows signs of internal activity. Records on the development of 88 eggs from deposition to hatching showed 3.3 days as the average egg period, the variation being 2.5-4 days. This is in close agreement with Maupas' statement ('00, p. 561) "les œufs mettent trois jours à parcourir leur embryogènie jusqu'à éclosion."

Immature Period

Length of Larval Stage.—In hen's egg cultures kept under the conditions of the laboratory, the larval period of 48 individuals of different progenies showed a variation of 8-17 days, although the usual variation was 8-14, 17 occurring but once and 14 being the next lowest number. The average larval period, based upon the above-mentioned records, is 10.1 days. In this paper, the larval period is regarded as extending from the moment of hatching to the deposition of the first egg, and not to the cessation of growth as will be discussed later. The appearance of the genital pore is an indication of maturity or closely approaching maturity but it was thought best to use the first oviposition as the final limit of the immature stage. Maupas ('00, p. 561) reported the larval period to be 10-11 days, at a temperature of 68° F. He also used the appearance of the first egg as evidence of the completion of larval existence.

In peptone cultures maintained under the more uniform temperature conditions of the underground cave described previously, the larval period of 10 individuals had a variation of 8-14 days, the average being 10.8 days. It will be noted that this agrees closely with the cultures reared under laboratory conditions and it appears that limited differences of temperature and the comparative culture values of peptone and yolk

of hen's egg had no striking effect on the time interval demanded for the attainment of sexual maturity.

Rate of Growth.—Careful, daily measurements of length of 8 individuals reared in peptone cultures under the cave conditions were made for about 22 days, a period of time well beyond maturity and the cessation of growth. It was noted that the period of growth and the larval period are not coterminal, but that growth usually continues 3-6 days after the production of the first egg. During this time, the increase in body-length has a variation of 0.019-0.076 mm., the average being 0.0527 mm.—about 10 per cent. of the average body-length of the completely formed individual.

At hatching, the larva is only 0.140-0.179 mm. long, the average of 16 specimens being 0.162 mm., and, from that time to the attainment of the full-grown condition, growth is continuous and approximately uniform. The growth curves of different individuals are very similar. The average daily increase in length was found to be 0.0258 mm., the variation being 0.019-0.03 mm. There is no evidence of a definite maximum daily rate of growth anywhere in the period and apparently growth, when completed, ceases almost as abruptly as it begins. The average growth period was approximately 16.3 days, with a variation of 14-18 days.

Length of Life

The complete life showed a variation of 20-61 days. Only 1 nematode lived 61 days, but 8 of 29 individuals lived 40-50 days, and 11 lived 30-40 days. The variation indicated above appears to be rather large and may have been due to some peculiar combination of factors in the different cultures, although all were given the same kind of food in approximately the same amount and kept under the conditions of the laboratory. Maupas ('00, pp. 561-562) records one individual which lived for approximately five months. The egg-laying period has already been discussed and it thus appears that if the first eggs of the parent be considered, a new generation can be produced in the cultures every 11-19 days, or about two generations per month.

REPRODUCTION IN DIPLOGASTER ÆRIVORA COBB

Source of Material

This nematode was first discovered in 1914 infesting the eggs of grasshoppers after they had been deposited in the ground. Specimens

were sent to Dr. N. A. Cobb who reported them as representing a new species. Later, Merrill and Ford ('16) found nematodes infesting the termite, *Leucotermes lucifugus*, which, when submitted to Cobb, proved to be the same species as the one found in grasshopper eggs and he described it under the name *Diplogaster ærivora*. Merrill and Ford made a special study of this nematode in relation to the termite host and published, in addition to Cobb's original description of the species, certain data on the reproduction and habits of this worm as observed in cultures. The present account includes the results of a more prolonged study of certain features of the reproduction, based upon continuous culture studies of more than three years.

Oviparity

Merrill and Ford ('16) have already pointed out the fact that males and females are continually present and that the males are functional. Cobb, in the same paper, described the morphological features of the two sexes. The writers have also found this species reproducing exclusively by the bisexual method. No evidence of hermaphroditism or parthenogenesis appeared, although evidence of such phenomena was sought continually.

Copulation

Copulation was easily studied in the cultures and the observations of the writers essentially confirm the brief account of Merrill and Ford ('16, pp. 125-126). Increased activity on the part of the male before mating was noticed. A female will copulate with several different males in a short time and the same is true of the behavior of the male towards different females. As will be shown later, observations indicate that a female must mate two or more times in order to produce fertile eggs throughout the adult life.

Fertile Eggs

The normal, fertile egg of *Diplogaster ærivora* is oblong, the average dimensions from forty-five measurements being 0.064 mm. and 0.035 mm., the variation being within 0.007 mm. The outer covering consists of a tough, membranous coat. Eggs are always deposited singly.

Infertile Eggs

An egg, which has seemingly never been fertilized and from which a nematode never develops, is designated as an infertile egg in these

studies. Such eggs are larger than the fertile ones, their dimensions being about 0.084 mm. and 0.043 mm. They are slightly variable in shape but are usually suboval. The envelope consists of a very thin membrane and encloses the finely granular, light colored contents. The general appearance of such eggs is sufficiently different from that of the fertile eggs to make it comparatively easy, with a little practice, to recognize them at sight in the cultures.

Relation of Copulation to Egg Production

An interesting relation between copulation and the production of both fertile and infertile eggs exists in this species. Fertile eggs were never deposited by a female previous to the initial copulation. A female, upon being mated, will, for a time, lay fertile eggs after which infertile ones will be deposited until another mating occurs. Upon being mated the second time, the female will again produce fertile eggs. For example, female No. 12 was mated at maturity, after which the male was removed from the cell. Twelve fertile eggs were deposited by this individual during the next 48 hours. During the following 60 hours, 8 eggs were extruded, all of which were infertile. After 6 days, this female was mated a second time and as a result, within 3 hours after copulation, fertile eggs were again deposited. Fifteen fertile eggs were laid during the following 3 days and 2 fertile eggs were found in the body of the female at death. Essentially, the same results were obtained in several different individuals maintained under similar conditions. On the other hand, female No. 88 was kept constantly exposed to males throughout her mature life and fertile eggs were uninterruptedly deposited until her decease. After the first mating of this female, eggs soon appeared and the number rapidly increased until well towards the end of the 2nd day at which time the maximum deposition of 19 eggs occurred. From this time on, there was a gradual reduction in the number of eggs produced until cessation occurred at the end of the 6th day, death ensuing about 24 hours later.

Occasionally, a female would completely exhaust her ability to deposit fertile eggs before any infertile ones were produced but, in the majority of cases, the approaching cessation of fertile egg production was indicated not only by the reduction in the number but also in the appearance of a mixture of fertile and infertile eggs leading to the final disappearance of the former. After a second or subsequent mating, the same phenomenon

usually occurred except in the reverse order, although, in some cases, there was an abrupt cessation of the infertile egg production after remating the female. Commonly, the maximum egg production follows the first mating, subsequent ones being followed by the deposition of a smaller number of eggs over a shorter length of time, but this is not a constant feature since a few exceptions appear in the records, as for example, female No. 81 was mated 3 times within a period of 11 days and the maximum production followed the third mating.

It thus appears that in order for the female of *Diplogaster ærivora* to attain her complete reproductive capacity, she must be mated at intervals throughout the egg producing period. One mating is insufficient but the reasons for this insufficiency are not definitely known at present. In all of the matings, the period of production of fertile eggs never exceeded 3 days and it may be that this represents the extent of the life of the spermatozoa after they have been transferred to the female. There is no reason for believing that the fertilization is other than *hysterogamic* (Lankester, '17, p. 505) and possibly fertile eggs cease to appear when all the spermatozoa of a single mating have been exhausted, but, if this be the case, there must be considerable variation in the number of spermatozoa transferred to the female at copulation since, according to the writers' records, the number of fertile eggs resulting from a single mating varied widely, as for example, initial matings resulted in 7-113 fertile eggs. Possibly the length of copulation, which is known to vary in this species, determines, in part at least, the number of male cells transferred.

Maupas ('00, pp. 586-587; 601-602) pointed out that there is a striking imperfection in the protandric hermaphroditic nematodes in which fertile eggs will be produced until the supply of spermatozoa is exhausted and then the same nematode will continue to produce infertile eggs in numbers 2 or 3 times greater than the fertile ones. The evidence seems to support the contention that this condition is the result of the failure of the ovo-testis to develop enough male cells. The infertile eggs invariably deteriorate. He also found that the occasional males of these hermaphroditic species sometimes fertilized the hermaphroditic individual after it had exhausted its own supply of spermatozoa. These cases may be comparable, if not homologous, to the condition in *Diplogaster ærivora*. Certainly, the disadvantage to the species is similar. Since all of the infertile eggs of *Diplogaster ærivora* deteriorate, the reproductive capacity of a single female is limited greatly unless males are ever present to fecundate her.

VIVIPARITY

At times, another form of reproduction occurs in the life history of this nematode in which living young appear within the body of the mother. This phenomenon existed many times in the cultures and was carefully studied. The first indication of this change in the normal reproductive procedure is the appearance of one or more very tiny nematodes within the body of the parent and inside of the uterus. These young move about actively and increase in size at a rapid rate. In time, they break their way through the wall of the uterus into the body-cavity of the mother and begin to actively attack her internal organs, soon causing her death. The young continue to feed upon the body contents of the dead parent until, in many cases, the whole interior is hollowed out, leaving nothing but the transparent cuticula within which the developing young wriggle about. From this empty parental cuticula, the young escape to the exterior and take up an independent existence in the surrounding nutrient medium. Approximately one-third to two-fifths of the complete growth may be attained within the parent. Merrill and Ford ('16, p. 126) observed this phenomenon in the parasitic strain of this species which they studied and they state that "Usually they were unable to escape, although instances were observed where they escaped through the genital pore of the mother." They also figure a dead female containing 14 young, all of about the same degree of development. It might be inferred from the above quotation that few of these young are able to complete their development, but the long observations on which this paper is based show that, in the strain from the grasshopper eggs, not only is the phenomenon fairly common but that the large majority of the young so developed escapes through the genital opening of the mother or through some rupture of her body-wall. Not only do such young complete their growth but they are perfect individuals and capable of reproduction. The number of young developing within the parent is variable. The writers have records of as many as 20 appearing at about the same time and also some evidence that even more may be so produced. On the other hand, the number may be as low as 3 or 4. These larvæ develop into both males and females and, since living young were never observed in females which had not at some time been exposed to males, it appears that they arise from fertilized ova and not from parthenogenetic ones.

Reproduction of this viviparous sort has been observed before in nematodes. Maupas ('00) found it to be a common occurrence in *Rhabditis elegans*, *Rhabditis caussaneli*, and *Diplogaster robustus* in which the eggs are not deposited as rapidly as they arrive in the uterus but tend to accumulate there, the delay causing them to be deposited ultimately in an advanced stage of development. Some of the young hatch in the uterus and are expelled along with the unhatched eggs but when the supply of spermatozoa is exhausted and the infertile eggs pass into the uterus, the young hatch, feed upon the infertile eggs accumulating there, grow, rupture the wall of the uterus, scatter in the general cavity of the mother, disorganize and devour the internal parts, and ultimately escape to the exterior. Pérez (Conte, '00b, p. 375) observed viviparity in *Rhabditis teres*, Conte ('00a; '00b) recorded it in *Rhabditis monohystera* and *Diplogaster longicauda*, and Southern ('09, pp. 93-94) described the occasional appearance of young within the body of the mother in *Rhabditis brassicæ*. Merrill and Ford ('16, p. 120) apparently found a similar occurrence in *Diplogaster labiata* in which "Occasionally a young nematode hatched within the body of a dead female," but no statement is made as to the ultimate fate of such individuals.

The factors initiating and influencing the appearance of this viviparity are not definitely understood. Conte ('00a) found that *Rhabditis monohystera* "*vivipare sur colle de pâte, est ovipare sur peptone*" and that the conditions of development are influenced by the nature of the nutritive medium. In another paper, Conte ('00b) points out that Maupas ('99) attributes the appearance of this viviparity to two causes, inanition and senility. Conte, however, found evidence that putrefaction in the medium was a cause, at least in the case of *Rhabditis monohystera*. "Tout en admettant avec lui que, dans certaines espèces, l'inanition et la sénilité amènent le parasitisme embryonnaire, je crois que ce phénomène peut être provoqué par d'autres causes et notamment, ches *Rhabditis monohystera*, par la putrèfaction du milieu. D'une façon générale, je pense qu'il est en relation avec un état morbide de la mère." In connection with his studies of the production of eggs or young in these nematodes, he finds it possible to distinguish the following stages which are closely related to the character of the nutritition:

Absolute oviparity—deposition of unsegmented eggs.

Relative oviparity—deposition of eggs undergoing segmentation.

Ovo-viviparity—deposition of gegs containing active embryos.

Viviparity—deposition of young which hatched in the uterus of the mother.

Embryonic parasitism—consumption of morbid mother by her offspring.

There is certainly a tendency in *Diplogaster ærivora* for this form of reproduction to appear towards the end of the reproductive period and while the parent may live and show body movements for a brief period after young appear within her body, her reduced vitality is apparent and is an accompanying feature, if not the causative one, of this phenomenon. However, the writers have evidence that age may not always be an accompanying factor, in fact, there is circumstantial evidence that any set of conditions which interferes seriously with the well-being of the female may lead to the appearance of living young within her body, even early in the reproductive period. It is therefore the opinion of the writers that this viviparity is the result of reduced vitality of the mother rendering her incapable of discharging the eggs. It also appears that eggs formed early in the reproductive period may hatch within the body as well as those produced at the end, and the writers have thus far discovered no positive evidence of any inherent predisposition of the last formed eggs for internal hatching.

There is no question that this appearance of young within the body of the mother constitutes a form of reproduction and that the resulting offspring are just as capable of continuing the species as those arising from hatching outside of the body. In reality, these two forms of reproduction are only superficially distinct and the use of the terms *oviparous*, *ovo-viviparous*, and *viviparous* is mainly one of convenience rather than one of exact distinction. Lankester ('17, p. 505) holds that "really all animals are viviparous, for the birth-product is a living thing whether it is a naked egg-cell or more or less advanced in development. The enclosure of the birth-product is a shell or case, which has given rise to the term 'oviparous' is not of any value as indicating the real degree of development of the young at birth, for in some cases unfertilized egg-cells, in others mere discs of developing embryonic cells (as in birds, etc.), and in yet other cases well-shaped young ranging from the early larva of some invertebrates up to the completely formed miniature of the adult, as in some of the shell-bearing snails, may be enclosed within an egg shell when 'laid' by the mother. There is accordingly no *general* importance

to be attached to the distinction between 'viviparous' and 'oviparous' animals." In this paper, these terms have been retained for the sake of convenience.

Proportion of Sexes

In the cultures studied by the writers, the total egg production of females varied widely, the average being about 55 eggs per female. The maximum observed was 196. In an attempt to determine the proportion of the sexes and the number of eggs and young per female, eggs selected at random were isolated, each in a separate culture. Females resulting from these eggs were mated at regular intervals throughout their lives. A complete daily record was kept of all eggs laid by these females and the sex of the resulting young determined by rearing them to maturity. Twenty-two females were studied in this way and, of the 437 resulting offspring, 182 developed into males and 291 became females. Since it was not possible to eliminate a certain mortality among the eggs and larvæ, the numbers given above can be regarded only as a general indication. They do show, however, that, in contrast to some of the bisexual species, the males are very common although the females are in the majority. These results agree, in general, with those of Merrill and Ford ('16, p. 126).

Records from the progeny of 22 females show that in the vast majority of cases both males and females appear in each progeny and also that in most of the progenies, females were numerically dominant. Since all the evidence indicates that the species is exclusively bisexual in its mode of reproduction, a sufficient number of males is demanded to maintain the generative processes, but since it was observed that a single male may and often does copulate with a considerable number of females, it seems probable that the smaller number of males indicates no important disadvantage in the multiplication of the species. In fact, the numerical dominance of the females coupled with fewer but sexually active males may possibly facilitate the production of a larger number of offspring, even if the locomotor ability is poorly developed.

Cobb ('18, p. 477), in discussing the comparative rarity of males in free-living nematodes, states that "There is reason to think that in some of the species the males are short-lived, and that this is the reason they are so rarely seen. The males are often so much smaller than the females that they are easily overlooked, or mistaken for young, so that in

such cases the rarity of the males may easily be overestimated." Since the length of life of the males and females of *Diplogaster œrivora* is virtually the same and since the length of the female exceeds the length of the male by only about one-fifth, it would seem that these features have not affected the observations on this species.

Rate of Growth

The young nematode, upon emerging from the egg, is active and moves about in much the same way as the adult. Its average length at emergence is about 0.238 mm. and the average diameter about 0.015 mm. A number of specimens were carefully studied in individual cultures for the rate of growth, all being reared under the same conditions and all living 12-23 days after hatching. Careful measurements were made at the time of emergence from the egg and at regular intervals of 24 hours thereafter throughout the life of each individual. Data were secured from 4 males and 5 females which were carried through their entire existence, each manifesting all of the activities of a normal individual and living for some time after growth had ceased. From the sedata, growth curves were constructed for the increase in length and diameter. These curves showed a striking similarity, not only in the different individuals of the same sex, but also in the individuals of the different sexes. Furthermore, the curves for the increase in length and the increase in diameter showed very close correspondence in every case. Growth begins immediately at hatching and continues uninterruptedly for a period, after which it ceases permanently. Composite graphs constructed on the basis of the individual growth curves showed that growth in both length and diameter ceases, on the average, on the 8th-9th day after hatching. In all cases except one, the variation from this average was very slight, the exception showing no growth after the 4th day. The average length of the males at the end of the growth period was 0.864 mm. and the diameter 0.052 mm., while the length of the females was 1.105 mm. and the diameter 0.068 mm. The length of life of the adult following cessation of growth varied with the individual from 6 to 15 days inclusive.

Length of Life

The length of the life of this nematode is, no doubt, subject to variation under different conditions. One series of eggs kept under average

temperature conditions of about 75° F., variation within 7°, required an average of 17.9 hours from the time of oviposition to hatching, the variation being 17-20 hours. A series of 29 individuals showed an average larval period of 3.75 days, the variation being 1.3-8 days. Twenty-three individuals of the same series had an average adult life of 13 days, the variation being 5-21 days. The average life of the individual outside of the egg was found to be about 17.1 days. Including the egg stage, the average life was approximately 18 days. Assuming copulation on reaching maturity, a generation can be secured in about 4.5-5 days. But very little difference was observed in the length of life of males and females.

SUMMARY

METHODS

1. Some of the free-living and semi-parasitic nematodes can be reared generation after generation in artificial media and their study thus facilitated. Two species, *Cephalobus dubius* Maupas and *Diplogaster ærivora* Cobb, were cultured continuously for over three years.

2. Cylindrical glass mounting-cells sealed with pure vaseline to ordinary microscope slides and covered with vaselined cover-glasses were found to be the most suitable containers for cultures. Methods of transference are described.

3. Of the numerous substances used as media, a very dilute solution of peptone, diluted yolk of hen's egg, and Pfeffer's synthetic agar were most extensively used. The eggs, ovaries, and body tissues of a number of insects were found to be favorable when properly prepared.

4. Unfavorable cultural conditions developed in stock cultures which were tightly sealed. Provision for ventilation was necessitated.

5. In starting cultures of *Diplogaster ærivora* from new stock taken from nature, mortality in the first generation was high but usually a few survived in the new medium and subsequent maintenance then became simple.

6. Temperatures above 80° F. are unfavorable, 90° F. and above proving fatal. These nematodes withstand a considerable fluctuation of temperature, e.g., 32° F. in 24 hours. Temperatures as low as 40° F. can be withstood, at least for a limited time. Optimum temperature conditions seem to be near 65-75° F.

CEPHALOBUS DUBIUS

1. Observations on long continued cultures of *Cephalobus dubius* involving many individuals and generations revealed no traces of males and reproduction seems undoubtedly parthenogenetic. If males ever appear, they must be extremely rare and develop under conditions of culture different in some unknown respect from those employed by the writers.

2. The egg-laying period has a variation of 6-44 days, average about 16 days. Oviposition, once initiated, continues uninterruptedly. The daily rate increases somewhat gradually to the middle of the period and then declines. As many as 27 eggs per day may be deposited. The total number of eggs per individual showed a variation of 33-285, average 139. Apparently, all eggs were capable of normal development.

3. Under cultural conditions, larvæ emerge from eggs 2.5-4 days after oviposition and reach maturity in 8-14 days.

4. The growth period and the larval period are not coterminal, growth usually ceasing 3-6 days after sexual maturity, during which time an increase in body-size of about 10 per cent. occurs. Larval growth is continuous and approximately uniform. The average daily increase in body-length is about 0.026 mm. Growth curves of different individuals are very similar.

5. The length of life has a variation of 20-61 days. Computing from the first eggs of a parent, a new generation can be secured in cultures every 11-19 days.

DIPLOGASTER ÆRIVORA

1. *Diplogaster ærivora* is bisexual and males are completely functional. One female may copulate with several males and males may behave similarly towards different females.

2. Both fertile and infertile eggs are deposited. Fertile eggs follow mating for a time, after which infertile eggs are laid until a second mating, after which the same sequence usually occurs. Constant exposure to males may completely prevent deposition of infertile eggs. Approaching cessation of fertile egg production is often indicated by the appearance of a mixture of fertile and infertile eggs. Usually, maximum oviposition follows the first mating.

3. All of the evidence indicates that the female must be mated at intervals throughout the egg producing period in order to fulfil her com-

plete reproductive capacity. The insufficiency of one mating is not definitely understood but it seems probable that infertile eggs appear upon the exhaustion of the spermatozoa received from a single mating. A similar imperfection in the reproductive ability appears in the protandric hermaphroditic nematodes and is explained by Maupas and others as due to exhaustion of the limited supply of spermatozoa.

4. Viviparity occurs from time to time. Young appear, first within the uterus, then later within the body-cavity of the mother, feeding upon her internal organs, untimately causing her death. In many cases, the interior is completely consumed, leaving nothing but the transparent parental cuticula from which the young escape to take up independent existence in the surrounding medium. Young so produced evidently originate from fertilized eggs and develop into both males and females capable of normal reproduction.

5. This form of viviparity has been observed by other workers and certain factors have been proposed as being responsible for the appearance of this phenomenon, namely, inanition and senility (Maupas), and putrefaction of the medium (Conte). The writers have incomplete evidence that it is the result of any factor or group of factors which reduce the vitality of the mother. Definite evidence was secured to show that, at least in *Diplogaster ærivora*, age is not always an accompanying feature but viviparity may appear at any time during the egg-laying period.

6. Females are numerically dominant, both in mass populations and in single progenies. Males, however, are common and very rarely absent, even in a single generation. Although fertilization is evidently necessary for any reproduction, it is probable that the smaller number of males is compensated for by their constant presence and their ability to copulate with several females.

7. Growth curves based upon daily measurements of length and diameter are strikingly similar for all individuals, irrespective of sex. Composite graphs showed the cessation of growth occurred on the 8th-9th day. The length of life following cessation of growth has a variation of 6-15 days..

8. The average length of life under cultural conditions is about 18 days. A new generation can be secured every 4.5-5 days. No marked difference in the length of life of males and females was observed.

LITERATURE CITED

Byars, L. P.
1914. Preliminary Notes on the Cultivation of the Plant Parasitic Nematode, Heterodera radicicola.
Phytopathology, 4:323-327.

Cobb, N. A.
1915. Proceedings of the Helminthological Society of Washington.
Journ. Parasit., 2:93-95.
1916. Proceedings of the Helminthological Society of Washington.
Journ. Parasit., 2:195-200.
1918. Free-living Nematodes. Fresh-water Biology, by Ward, H. B., and Whipple, G. G., pp. 459-505.

Conte, M. A.
1900a. De l'influence du milieu nutritif sur le développement des Nématodes libres.
C. R. Soc. Biol., 52:374-375.
1900b. Sur les conditions de ponte des Nématodes.
C. R. Soc. Biol., 52:375-376.

Johnson, G. E.
1913. On the Nematodes of the Common Earthworm.
Quart. Journ. Micr. Sci., 58:605-652.

Lankester, E. R.
1917. The Terminology of Parthenogenesis.
Nature, 99:504-505.

Martin, A.
1913. Recherches sur les conditions du développement embryonnaire des Nématodes parasites.
Ann. Sci. Nat., Zool., (9), 18:1-151.

Maupas, E.
1899. La mue et l'enkystement ches les Nématodes.
Arch. Zool. exp., (3), 7:563-628. 3 pls.
1900. Modes et formes de reproduction des Nématodes.
Arch. Zool. exp., (3), 8:463-624. 11 pls.

Merrill, J. H., and Ford, A. L.
1916. Life History and Habits of Two New Nematodes Parasitic on Insects.
Journ. Agr. Research, 6:115-127.

Metcalf, H.
1903. Cultural Studies of a Nematode Associated with Plant Decay.
Trans. Am. Micr. Soc., 24:89-102.

Oliver, W. W.
1912. The Cultivation of an Ectoparasitic Nematode of a Guinea Pig on Bacteriologic Media.
Science, (n.s.), 36:800-801.

Osborn, H.
1898. The Hessian Fly in the United States.
Division of Entomology, U. S. Dept. Agr., Bull. 16, n. ser. 58 pp. 2 pls.

Potts, F. A.
1910. Notes on the Free-living Nematodes.
Quart. Journ. Micr. Sci., 55:433-484.

Southern, R.
1909. On the Anatomy and Life-history of Rhabditis brassicæ, n. sp.
Journ. Econ. Biol., 4:91-95. 1 pl.